**Purpose:** synthesize a gene of interest from scratch

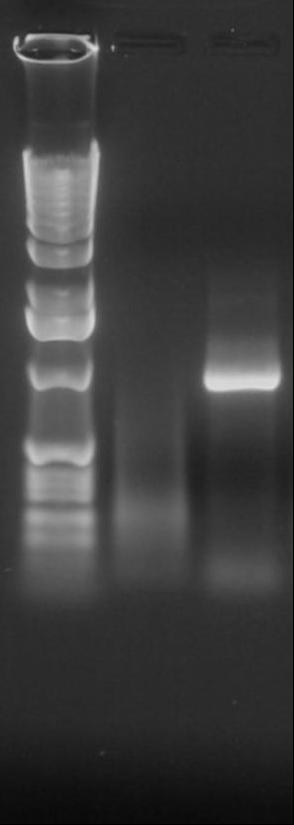
In this lab, your objective is to clone your assigned gene using overlap extension PCR with the oligos designed using DNAworks. Then you will amplify full-length copies of your gene using forward and reverse primers. You will analyze your PCR products using agarose gel electrophoresis and ligate your PCR product with an intermediate vector, cloning vector, which will allow you to analyze the cloned gene and to determine if the DNA sequence is correct.

**Primer Dilutions:** You will need to make an Oligo Mix consisting of all of your primers that were ordered after designing them in DNA Works. These primers arrive in deep well plate format from IDT. The stock concentrations are 100 uM.You will need to make a single mix that will contain a final concentration of 1 uM for each primer. It is very important to not cross- contaminate your primers with someone else’s. Use a marker to label the plate and show which primers are yours.

* Make a new microcentrifuge tube labeled with the Date, Your Initials, Gene name, and ‘Primer Mix’.
* Add autoclaved Nanopure water to the tube. The amount of water will need to be equal to 100 - # of primers you have.
  + For Example, if you have 12 primers for your gene, you would need 88 ul of water.
* With a 200 ul tip – gently mix the contents of each primer in the deep well plate
  + **Be sure to change tips between each well!**
* Then, go back and remove 1 ul of each stock primer from the plate and add to the new ‘Primer Mix’ tube

NOTE: If you have more than 1 oligo mix for your gene or gene part because you have to synthesize your gene in pieces, please set up one primary PCR reaction for each oligo mix.

**I. Primary PCR**

Your first step PCR is to fill the gaps between your oligos and piece together a full length DNA for the gene of interest.

1o

PCR

2o

PCR

1o PCR

**Figure 1a**: This is a schematic diagram of some of the products that you will get from your 1o PCR reaction. If you run a gel of your 1o PCR reaction, you will basically get a smear as seen in Figure 2. Some of your 1o PCR product will contain your full-length gene and this will be amplified using a forward and reverse primer to result in a band that will correspond with the length of your target gene.

**Figure 1b**: The 1o and 2o PCR reactions ran on a 1% agarose gel with the 1o PCR reaction highlighted.

**PCR Materials**: All KOD PCR reagents are found in a box labeled “KOD PCR reagents” in the top loading -20oC freezer.

**Primary PCR:**

5 l 10X rxn buffer

3 l 25 mM MgSO4

5 l 2 mM dNTPs

1 l Template/Primers = 1 M oligo mix

1 l KOD hotstart Polymerase (1U/l)

35 l sterile dH2O

50 l Final Volume

**Thermocycler cycling conditions:**

95oC 2 min

95oC 20 sec

58oC 10 sec

20 cycles

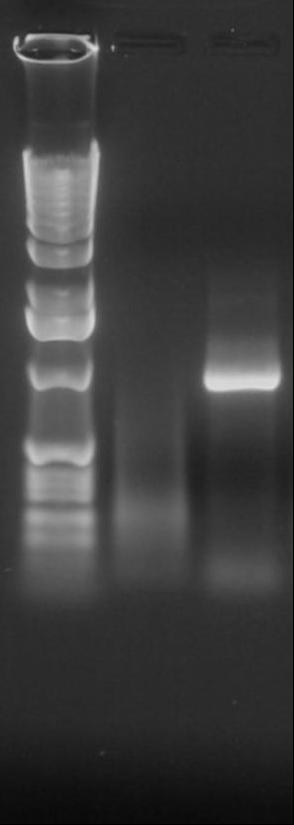
70oC 20 sec

70oC 2 min

**You will use ~1 ul of this sample for the next step. Save the other half in -20OC and label it well.**

**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**II. Secondary PCR:**

****

Reverse primer w/

lagger

1o

PCR

2o

PCR

Forward primer w/

leader

2o PCR

**Figure 2b**: The 1o and 2o PCR reactions ran on a 1% agarose gel with the 2o PCR reaction highlighted.

**Figure 2a**: This is a schematic diagram of the final product that you will get from your 2o PCR reaction. The Forward and Reverse Primers will only amplify the full length gene from the pool of DNA fragments in your 1o PCR reaction. This will show up as a strong band corresponding to the size of your CDS (coding DNA sequence) when you run your 2o PCR reaction on an agarose gel.

**NOTE:** If more than one primary PCR reaction was set up for your gene or gene part, please add 1ul of each primary PCR reaction into your secondary PCR reaction. **You would need to adjust the volume of sterile H2O accordingly to keep the final volume at 50l.**

**Secondary PCR:**

Thermocylcer cycling conditions similar as primary PCR but the **number of cycles is increased to 30**.

5 l 10X rxn buffer

3 l 25 mM MgSO4

5 l 2 mM dNTPs

1 l Template = primary PCR reaction (if more than one primary PCR reaction was set up, use 1ul from each primary PCR reaction)

1 l 20 M F primer i.e. first primer from DNA Works

1 l 20 M R primer i.e. last primer from DNA Works

1 l KOD hotstart Polymerase (1U/l)

33 l sterile diH2O (adjust this volume if more than one primary PCR reaction was added to mix)

50 l Final Volume

**Run half of your sample on an agarose gel to verify amplification. Save the other half in -20OC**

To confirm that your PCR worked and that you made your oligo mix correctly, you need to run your 1o PCR and 2o PCR products on a 1% agarose gel with DNA ladder. Your 1o PCR product should look like a smear. This smear will probably not be the same size as your full length CDS. If there is no smear – it is ok if your 2o PCR product has a distinct band that corresponds to the size of your gene.If so, then you can proceed to set up a **PCR squared** reaction (which is a large-scale amplification reaction) using the secondary PCR reaction as your DNA template.

**Consult with the mentor or RE if you do not get a smear or a band.**

**\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_**

**III. PCR2 squared reaction (30min to set up and 1.5h for PCR)**

* This step is optional. Based upon your yields from 2O PCR in the gel – you can skip this and go directly to PCR cleanup. If your PCR cleanup yields low then you can come back and do PCR2 squared.

**PCR2 squared: Use the same program as Secondary PCR (i.e. 30 cycles). Measure the following reagents into a PCR tube sitting in a PCR tube rack on ice.**

Master mix for PCR squared reaction

20 l 10X rxn buffer

12 l 25 mM MgSO4

20 l 2 mM dNTPs

8 l Template = secondary PCR reaction

4 l 20 M F primer

4 l 20 M R primer

4 l KOD hotstart Polymerase (1U/l)

128 l sterile diH2O

200 l Total volume

After the master mix above is made, vortex the tube to mix well and make 50ul aliquots into 3 additional PCR tubes so that you have a total of 4 PCR tubes with 50ul each of the PCR squared reaction.

Store all PCR reactions in your box in the -20oC freezer. Or you can proceed to next step.

**PCR Cleanup**

Use Sigma PCR cleanup kit in red box.

NOTE: max starting volume is 100 ul (or 10 ug), but you should not make multiple preps, rather run each prep through the same column in the first steps. This will increase your yield and concentration.

**Elute** in 50 ul only (not 100)

**Elute** with Tris HCl (not TE – tris EDTA) because the EDTA may affect ligation efficiency.

Alternatively, **Gel Extraction**

Run your PCR product on a 0.9% agarose gel (if making 100 ml, then use 0.9 grams of agarose)

Use the large wells (6 well combs)

Use 100 bp ladder

Excise all proper bands of the PCR product with a razor blade and transfer to a tube.

Use Sigma Gel Extraction kit (another red box) to remove agarose from PCR product

NOTE: may need to use PCR cleanup afterwards if getting bad reads on nanodrop from contaminating reagents

**Nanodrop**

Determine concentration of cleaned PCR product via spectrophotometry. Save printout of graph and note 260/280 and 260/230 ratios along with final concentration.